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CHANGES IN NADPH OXIDASE (NOX) PROTEIN ISOFORMS AND DOWNSTREAM REACTIONS AS FUNCTION OF TIME AND OVERPRESSURE IN BLAST TBI

by

Smit P. Shah

Blast-induced Traumatic brain injury (bTBI) is a leading cause of morbidity in soldiers on the battlefield and training sites with long-term neurological and psychological pathologies. Among many pathological sequela of blast TBI, oxidative stress has been identified as a major factor contributing to the pathophysiology of bTBI. Recent studies have demonstrated activation of oxidative stress pathways following blast injury but their distribution among different brain regions as function of post injury time and Blast over pressure (BOP) have not been explored. The present study examines the protein expression of NADP oxidase (NOX) isoforms 1 & 2, corresponding superoxide production, a downstream event of NOX activation as well as the extent of lipid peroxidation adducts of 4-Hydroxynonenol (4-HNE). Based on these results, in the present study, we asked: 1) whether NOX protein levels change as a function of different overpressures in bTBI? 2) Whether such changes follow a temporal pattern? and 3) what are the consequences of NOX protein changes on the downstream events including superoxide production and lipid peroxidation of proteins. The Brain injury was evaluated at 4, 24 hours and 7 days and at 130, 180 and 240 kPa blast overpressures. Results showed that NOX isoform expression display a bi-phasic response wherein, its expression did not change at 130 kPa, whereas a significant increase was first detected in animals exposed to 180 kPa BOP which displayed a further increase at 240 kPa.

Examination of temporal changes in NOX protein levels again displayed a biphasic response with a significant increase at 4h post- injury which peaked at 24 h and completely restored to that of control levels at 7 days post-injury. Blast exposure also resulted in increased superoxide levels in different brain regions as well as changes in lipidid peroxidation product 4hydroxynonenol (4HNE) protein adduct formation. Collectively, our results demonstrate that NOX isoforms are upregulated in different brain regions as a function of different overpressures in bTBI and temporally display a biphasic response. Oxidative stress therefore appears to be a higher risk factor in the pathogenesis of bTBI.

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by

Smit Prakash Shah

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APPROVAL PAGE

CHANGES IN NADPH OXIDASE (NOX) PROTEIN ISOFORMS AND DOWNSTREAM REACTIONS AS FUNCTION OF TIME AND OVERPRESSURE IN BLAST TBI

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After an intensive period of almost a year, today is the day: It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this dissertation has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality worldwide. There are about 250,000 cases of TBI annually occur in USA which there are about 50,000 severe cases of TBI resulting in death. TBI has been broadly classified into two categories, namely blunt and blast injuries based on the type of impact occurring to the skull and the brain [1]. Blunt TBI typically include closed head injuries wherein external damage to the skull is usually minimal which frequently results from falls, sports-related head injuries [2, 3]. Other form of blunt TBI usually results from penetrating injuries which usually causes damage to the to the skull by sharp objects such as knife or fast moving bullets causing damage to both skull which penetrates deep into the brain [1]. Among overall TBI hospitalizations, about 50% of cases are due to falls, while automobile accidents and the impact by striking objects comprise about 30% [5].

Another important form of TBI is the blast-induced TBI (bTBI) which is the most prevalent form of brain injury in soldiers due to growing number of use of improvised explosive devices (IED's) by insurgents on both military and civilian population [6-8]. As depicted in Figure 1, in terms of the nature of injury, blast injuries are broadly categorized into 4 forms: a). Primary injury caused by the direct mechanical forces generated by shock waves; b). secondary injuries resulting from the hitting and penetration of sharp objects through the skull into the brain; c) tertiary injury including impact of the other objects and sudden falling on the ground and d) quaternary injury due to heat generated by the explosion and exposure to toxic gases [9-11].

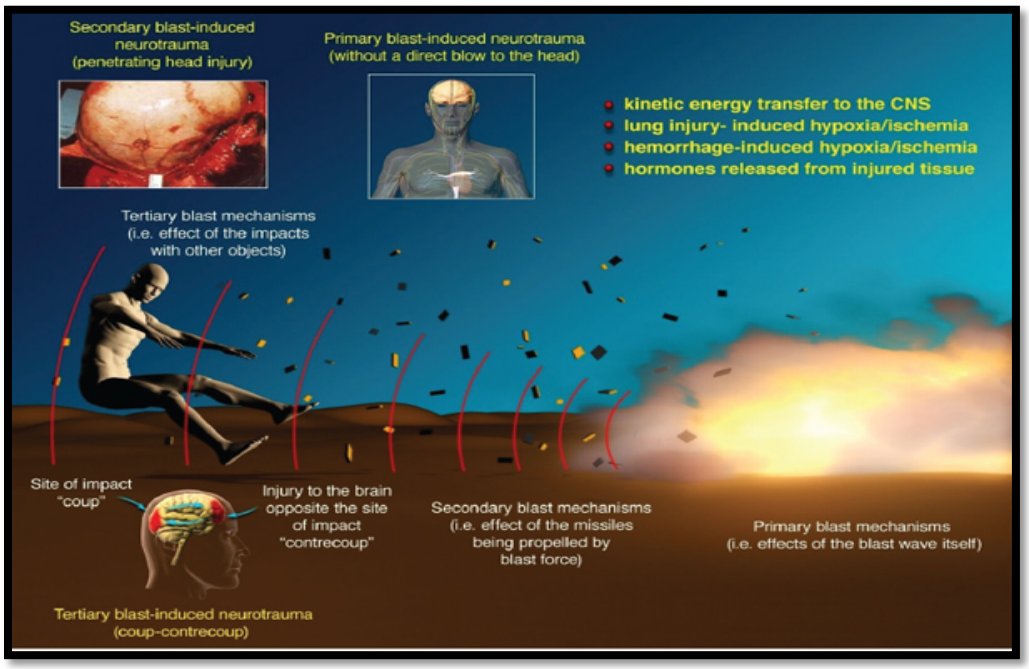

Figure 1.1: Schematic diagram depicting various categories of injuries in blast TBI. Source: [4].

Several animal models have been developed for TBI, particularly those for blunt TBI which include weight drop injury (WD), control cortical impact injury, (CCI), fluid percussion injury (FPI) [12]. The WD model mimics closed head injuries frequently associated with falls and sports concussions which are very common form of TBI worldwide [2, 3]. In WD model, a known quantity of weight (usually metal rod) is dropped onto animal head from a predetermined height, which does not cause skull damage but will impact brain [14]. Another model namely controlled cortical impact (CCI) uses a solid impactor to damage exposed dura which usually involves the use of a pneumatic or electrochemical device [15]. CCI injuries typically manifest cortical tissue loss, axonal injury, BBB dysfunction, and subdural hematoma [15-18]. Another most frequently used model of TBI is the fluid percussion injury (FPI) model, an invasive

procedure in which a craniotomy is performed to expose a portion of the dura. The injury is created by passing a fluid pulse (sterile saline) which is produced by dropping a pendulum from a certain distance [19].

While several animal models of blast TBI have been currently in use, majority of these models do not truly represent the properties of blast-induced TBI in battlefield conditions. The blast injury model (shock tube) developed by Chandra's group [20-23] represents an ideal model to understand the properties of shockwaves and their impact on the pathophysiology of bTBI (see section 2.1, Chapter 2).

1.1. Neuropathology of Blast TBI

Although the pathophysiology of bTBI continues to be expanding, thus far there is only a limited understanding of how blast waves interact with the brain and cause injury. Therefore, exploration of the primary impact of shockwaves on different regions of brain, the nature of impact and the outcome of the pathology is necessary to better understand the injury pathways.

Several pathophysiological outcomes have been identified in mild, moderate and severe forms of blast TBI in animal models. These include the epileptogenic seizures in mild and moderate bTBI, brain edema, cerebral vasospasm, diffused axonal injuries, glial cell activation, and neurodegeneration (for review, see [24]).

Among many pathological events identified in bTBI, major mechanisms believed to be responsible for neuropathological outcomes in bTBI include breakdown of blood brain barrier (BBB), neuroinflammation and oxidative stress [13, 25-27].

Studies have shown disruption of BBB integrity in animals exposed to blast injury [28-31, 32. Recent studies by Kuriakose et al report increased BBB permeability as early

as 15 min in animals exposed to moderate (180 kPa) blast injury {Kuriakose, 2018 #4501].

Neuroinflammatory responses have also been identified in bTBI. Accordingly, activation of microglia, increase production of inflammatory cytokines, activation various chemokine pathways, reactive gliosis, phagocytic responses have been reported in mild to moderate level of blast TBI [27, 33-37].

Among many pathological factors associated with either primary mechanical injury or secondary biochemical cascades, oxidative stress has been shown to play a major role in various models of TBI [38, 39]. The main inducers of oxidative stress are reactive oxygen species (ROS) which include superoxide ($O2\cdot-$), hydroxyl radical ($HO\cdot$), and hydrogen peroxide (H_2O_2) [40, 41]. ROS are normally produced in several metabolic reactions, including redox-reactions (oxidation/reduction), oxidative phosphorylation and in a normal process of electron transport chain reactions. There are a number of enzymes that produce free radicals during their catalytic reactions, which include the NADPH oxidase family, cytochrome P450 (CYP450), cyclooxygenase (COX), lipoxygenase (LOX), and xanthine oxidase (XO).

Previous studies have demonstrated that reactive oxygen species (ROS) such as the superoxide radicals and nitric oxide can form peroxynitrite, a powerful oxidant that impairs cerebral vascular function after bTBI [27].

1.2 Reactive Oxygen Species.

The major inducer of oxidative stress are reactive oxygen species (ROS) which include superoxide (O2·−), hydroxyl radical and hydrogen peroxide (H_2O_2) all of which have inherent chemical properties that confer to different biological pathways. Reactive oxygen species (ROS) are passively released from mitochondria or actively produced from enzymatic sources, including NADPH oxidases. Although ROS could mediate physiological functions by acting as second messengers, excessive production of ROS damages proteins and organelles and leads to detrimental consequences in the heart. Reduction and oxidation (redox) is an important mechanism of post-translational modification of signaling molecules that regulate a wide variety of functions, including

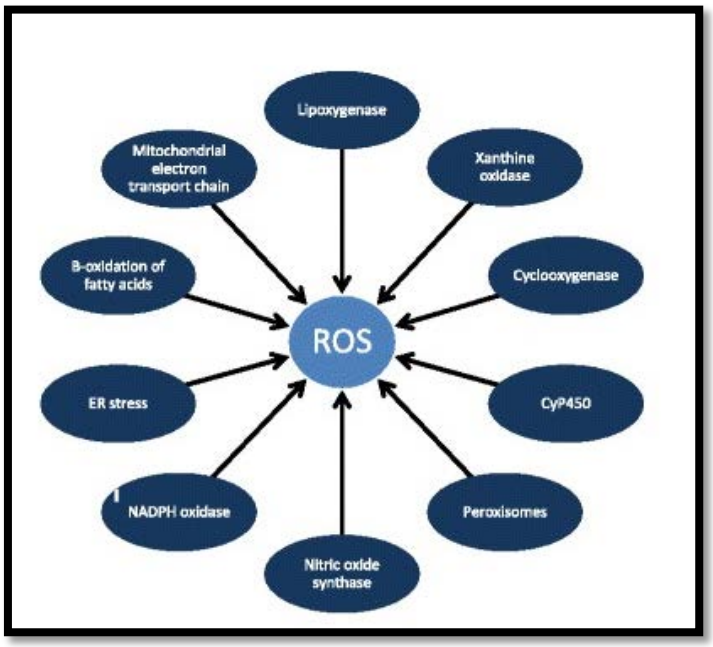

Figure 1.2: Schematic diagram depicting various biochemical pathways that produce reactive oxygen species (ROS) in biological systems. Source: Ma et al. Molecular Neurodegeneration (2017) 12:7 DOI 10.1186/s13024-017-0150-7

growth, death, differentiation, contraction, and metabolism in cardiomyocytes. Oxidative stress is a major cause of myocardial damage during ischemia/reperfusion,

pressure overload, and aging. In order to reduce excessive amounts of ROS and reverse oxidative post-translational modification of proteins, cells utilize antioxidants including the thioredoxin system. ROS is often associated with the principle of oxidative stress which suggest ROS induced pathology. The main cellular source of reactive oxygen species is mitochondrial respiratory chain and active NADPH oxidase family. The following figure illustrates various cellular sources of ROS.

1.3 NADPH Oxidase

Significant source of superoxide in brain is NADPH oxidase, a multi-subunit enzyme that catalyzes the reduction of molecular oxygen and oxidation of NADPH to generate superoxide radicals (O_2).There are different types of NOX isoforms in brain which include NOX1,NOX2 and NOX4 and they are highly depend on cell type. Neurons express both NOX1 and NOX2, microglia are enriched with NOX2, while only small amounts of NOX isoforms were identified in astrocytes [44].

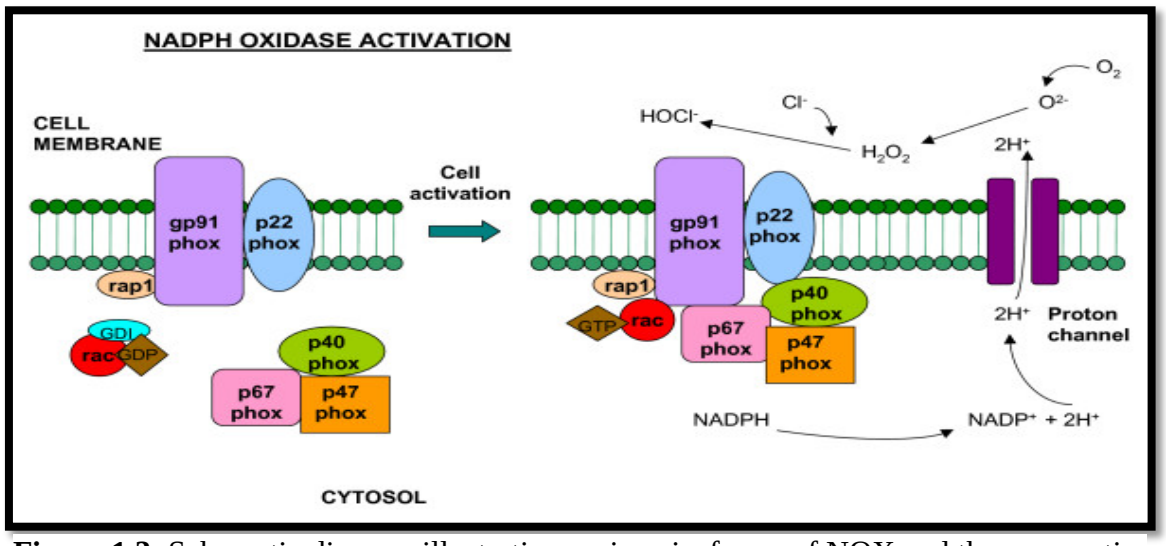

Figure 1.3: Schematic diagram illustrating various isoforms of NOX and the enzymatic reaction of NOX to produce the superoxide radical. Source: Chronic Granulomatous Disease; fundamental stages in our understanding of CGD Tracy Assari published 21 September 2016

NOX comprises subunits that are both plasma membrane-bound (cytochrome b_{558}, comprised of $p22^{phox}$ and $gp91^{phox}$) and cytoplasmic ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), which spans across the lipid bilayers [42, 43].

Extensive experimental evidence suggests NOX plays a significant role in the pathophysiology of various forms of TBI. NOX has been shown to be upregulated in a brain in controlled cortical impact model of trauma [45] and closed head injury models [46, 47].

While studies establish a primary role of NOX1 in the pathophysiology of various forms of TBI, no studies have been performed to determine the spatial and temporal resolution of NOX family of enzymes in the brain and their role in the pathophysiology of bTBI. A recent report from our laboratory has shown increased levels of NOX isoform expression in acutely (4h) after single blast injury at moderate overpressure (180 kPa). Such increase in NOX protein was found to be more localized in hippocampal neurons compared to astrocytes and microglia [13].

1.4 Hypothesis and Rationale

Based on the existing literature on the NADPH oxidase-mediated oxidative stress in bTBI, we identified several gaps in the knowledge in this area of research. We therefore raised the following questions: 1). How does NOX expression vary as a function of blast over pressure (BOP)? ; 2) How does NOX expression varies as a function of time post-injury at a given BOP?;3). Is NOX expressed differentially across different cerebral regions at a given time point and BOP?; 4). Do changes in NOX expression correlate

with superoxide production?; 5). Does superoxide production lead to downstream alterations such as lipid peroxidation of proteins in blast injury?. Based on these ideas, we ***hypothesized*** that that there will be a temporal and spatial variation in the expression of NOX as a function of different overpressures in blast TBI.

CHAPTER 2

MATERIALS and METHODS

2.1 Animals

Adult 10-week-old male Sprague-Dawley (Charles River Laboratories) rats weighing 320–360 g were used in all the studies. The animals were housed with free access to food and water in a 12-h dark-light cycle at 22°C. All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals and were approved by Rutgers University Institutional Animal Care and Use Committee (IACUC) before experiments. A total number of 64 rats were used for different types of experiments including immunostaining, Western blotting and *in vivo* measurement of superoxide. Rats were divided into two groups: Control and Blast-injury group, the latter group is subdivided into 3 categories based on the animals exposed to varying blast overpressures (130 kPa; 180 kpa and 240 kPa).

2.2 The Shock Tube

The shock tube located in Prof. Chandra's laboratory is a well validated tube that is capable of producing field validated shock waves. It contains a 9" square cross section with adjustable volume breech, variable length section, 6 meter long test section. Shock waves are produced using a compressed air or helium that passes through the volume

breech section which is separated by Mylar membranes. Placing membranes with different thicknesses will allow to achieve different overpressures. The pressures inside the breech section is continuously monitored by WIKA A-10 sensor (0-340 atm range) and the burst pressure was recorded for all the tests (Figure 2.1). Pressure waves are monitored by pressure sensors place at different area along the shock tube and the data is recorded at 1 1.0 MHz frequency with an acquisition time of 200 msec (Figure 2.1).

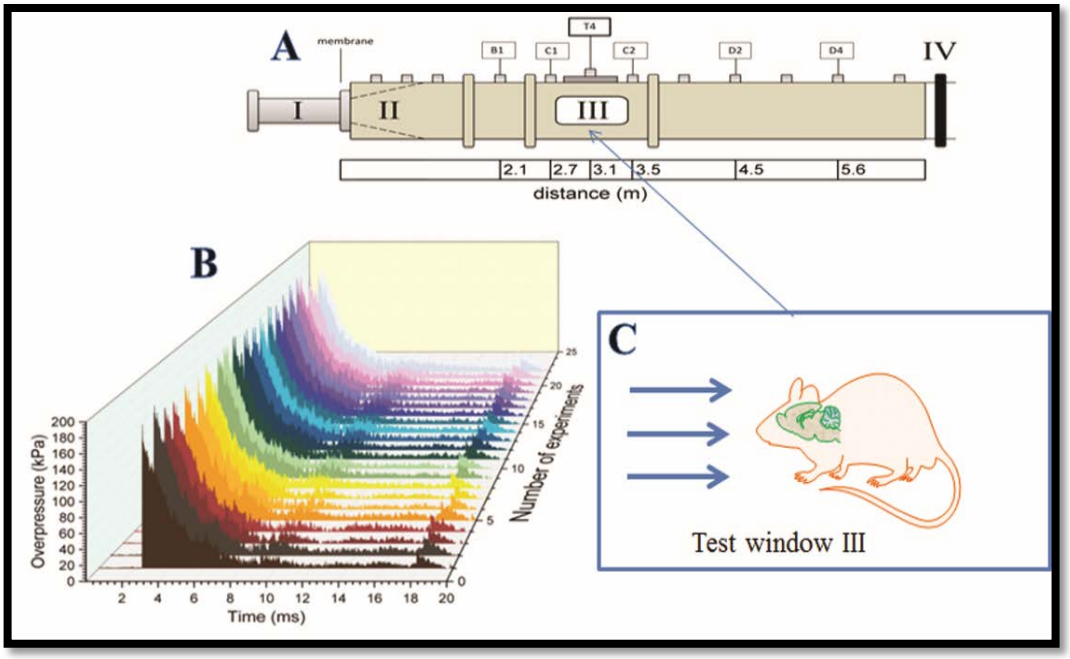

Figure 2.1: Schematic depiction of the shock tube. (*A*) Schematic of 9 in X 9 in square 30 feet long shock tube with section I-Breech with high pressure helium gas separated from section II by different thickness of mylar sheets that generate pure shock wave in section III where the specimens are located. Section IV is past the section and is a design requirement; the pressure-time cycle is identical to live fire tests with actual C-4 (or TNT equivalent) explosives at specified stand-off distance. (*B*) Composite of actual experimental profiles that generate 180 kPa with only about 5 kPa variation in peak pressure and less than a millisecond in duration. The front of the pressure rise indicates shock wave conditions. (*C*) Schematic of rodent model in prone facing the shock front. The shock travels in the rostral-caudal direction traversing pre-frontal cortex, striatum, hippocampus, thalamus, visual cortex and cerebellum within a period of a millisecond with minimal attenuation of pressure loading. Source: *Rama Rao K.V.……Chandra N: [13]*

2.3 Blast Injury

Rats used in the present study were exposed to a single blast wave in the shock tube. Before exposing the rats to shockwave, all rats were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (10 mg/kg) at 10:1 ratio administered via intraperitoneal injection. Rats were subjected to a single exposure to 3 different peak overpressures): 130 kPa (18.8 psi), 180 kPa (26 psi) and 240 kpa (35 psi) peak overpressure. All rats were mounted in the middle of the shock tube (2.8 meters from the breech, and 3 meters from the exit) in a prone position, i.e. were strapped securely to the aluminum plate using a cotton cloth wrapped around the body [48]. The cloth provides no protection against the shock wave, but prevents any excessive head motion [49] (Figure 2.2). Sham control rats received anesthesia and noise exposure but without blast exposure, i.e. anesthetized animals were placed next to the shock tube, and then a single blast was fired. The entire blast procedure was recorded with a high-speed video recording to capture any substantial head and body motion during the blast so as to exclude the impact of tertiary bTBI. Following blast injury, animals were monitored closely for any signs of trauma-related distress (e.g., apnea).

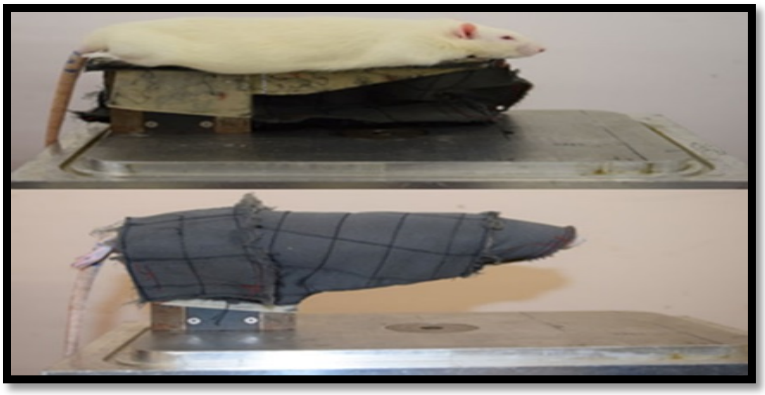

Figure 2.2: Anesthetized rat strapped and secured on the surfboard before mounting onto the test chamber. Strapping the animal prevents the head or body motion during the propagation of the shock wave.

2.4 Western Blots

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize. In our study Western Blots are used to identify changes in the protein adduct formation of lipid peroxidation product 4-hydroxynionenol (4HNE) expression in rat whole brain at various Blast over pressure and time points. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

In the present study for Western blot analysis, whole brain homogenates from control and animals exposed to blast injury of different over pressures were used. Animals were anesthetized with ketamine/xylazine mixture (100mg/kg and 10mg/kg respectively), transcardially perfused with PBS. Following perfusion with PBS, brains were excised from the cranial vaults, the whole cerebral hemispheres were homogenized in ice-cold conditions using CellLytic-M (Sigma) using sonicator with probe amplitude set to 45%. Samples were then centrifuged at 14,000× g at 4^0C. The protein concentration in the samples was estimated by bicinchoninic acid (BCA) method (Thermo Scientific,

Rockford, IL). Prior to loading the samples in the gel, the protein concentration in each sample was normalized to equal volumes containing either 10 µg or 20 µg depending on the protein to be probed with primary antibody. Every caution was taken to ensure that equal volume containing equal amount of protein in each sample was being loaded into the gel. Electrophoresis was performed using 4-15% SDS-PAGE gradient gels (Bio Rad). Proteins separated according to their molecular size were then transferred onto PVDF membranes using Turbo Protein Transfer instrument (Bio Rad Laboratories) using manufacturer's instructions. Membranes were blocked with 5% milk dissolved in Tris-Buffered saline containing 0.1% Tween-20 (TBS-T) and incubated overnight at 4^0C with 4HNE (Abcam, Cambridge, MA) at a dilution of 1:500. Bands were visualized using Western Pico Chemiluminescence Substrate (Thermo Scientific) on Chemi Doc Imaging System (Bio Rad Laboratories). Once probed with antibody for protein of interest, in this instance, 4HNE, PVDF membranes were incubated in stripping buffer (Restore™ ThermoFisher Scientific) using the instructions provided by the manufacturer.

Membranes were reblocked with 5% milk solution and probed with β-actin, a housekeeping protein to observe any variations in during loading of protein samples into the gel. For densitometry quantitation of western blots, images were digitized using a BioRad GS800 calibrated densitometer, and analyzed with BioRad Quantity One software. The arbitrary densitometry values obtained for each protein band in each lane was normalized to corresponding actin protein band and final values were expressed as % change over control. A diagram showing various steps involved in the Western blot analysis is presented in Figure 2.3.

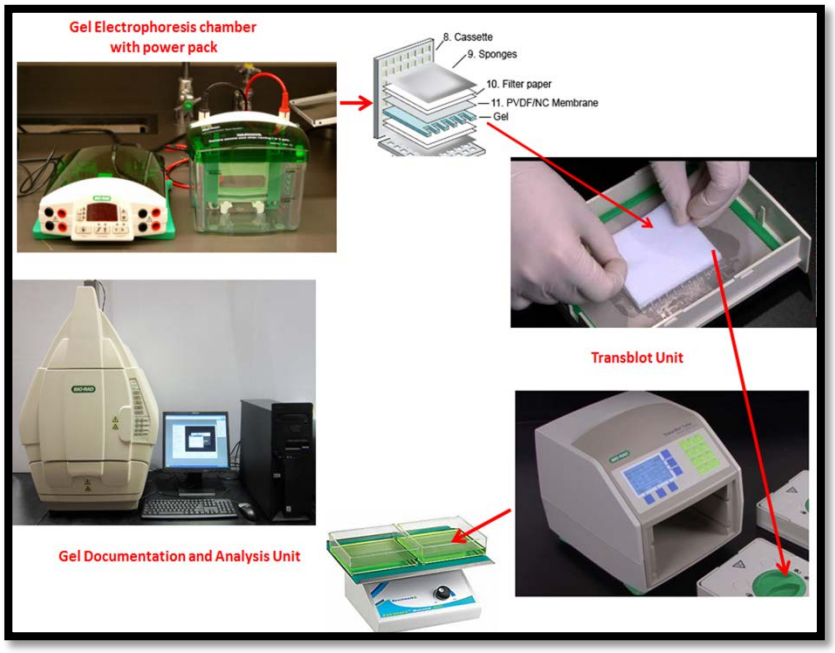

Figure 2.3: Gel electrophoresis units, gel transfer system (Transblot Turbo mini) and gel analysis system used in the present study.

2.5 Immunofluorescence Staining

Immunohistochemistry (IHC) staining is performed to detect a protein of interest in a tissue section obtained by fixation methods. The detection of a specific protein (antigen) in tissues is accomplished using antibodies that recognize a target protein and antibody-

antigen complexes that are visualized via chromogenic, radioactive or fluorescent substrates. For immunofluorescence staining, secondary antibodies tagged with fluorescent compounds as flurescence isothiocynate (FITC) or texas red or rhodamine that are visualized by fluorescent microscopes equipped with filter cubes that can regulate the excitation and emission wavelength.

There are a variety of techniques for sample preparation and visualization, and the method used should be tailored to the type of specimen under investigation and the degree of sensitivity required.

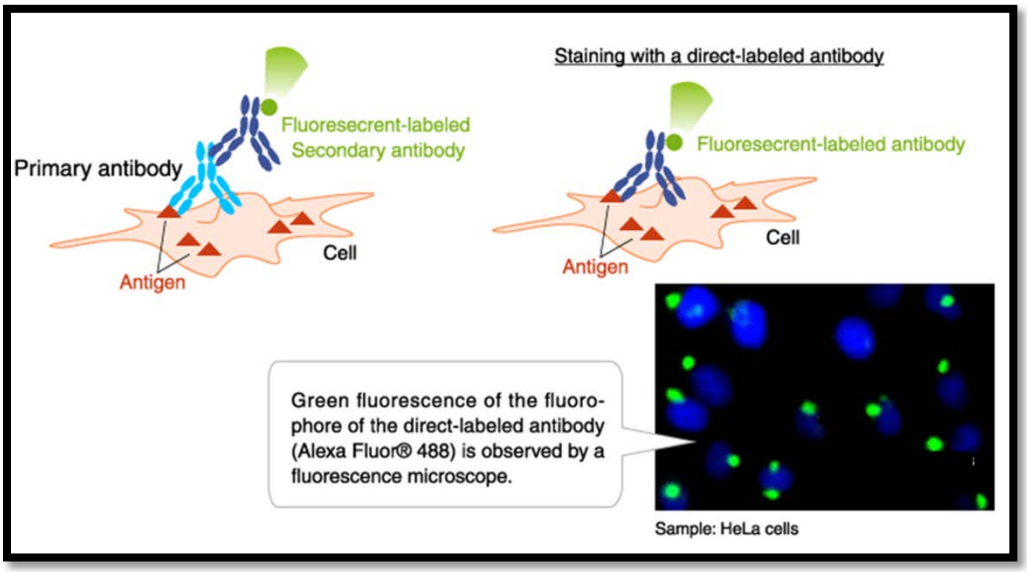

Figure 2.4: Schematic diagram depicting the immunostaining procedure. Using Alexa Flour secondary antibodies is extremely useful when performing a double immunofluorescence staining for which two Alexa Flour conjugated secondary antibodies can display two different fluorescent colors. *Source: MBL Inc. Woburn, MA*

In order to evaluate the spatial changes of NOX1 and NOX2 proteins as a function of varying BOP and time post injury, we performed immunofluorescence studies of two isoforms of NOX in frontal cortex, hippocampus and thalamus. Briefly, sham and TBI animals were transcardially perfused with PBS followed by 4%

paraformaldehyde (PFA). After perfusion, the brains removed from cranial vaults and incubated in 4% PFA for additional 48 h and cryoprotected by immersing in 30% sucrose. A thin 20 µm sections were prepared using Leica VT 1000S semi-automatic vibratome. In brief, brains were removed from sucrose solution and mounted onto specimen disc with glue and then the plate introduced into buffer and sections were cut at 20 µm thickness using blade with the speed and frequency of vibrations set optimum. The cut sections were mounted onto microscope slides (Fisher Scientific, Nazareth, PA), air dried and stored at -20^{o}C until immunostaining.

Glass slides prepared from 4 individual animals in each group were washed with 10 mM phosphate buffered saline (PBS), fixed in ice-cold methanol (100%) solution for 10 minutes at −20 °C. The tissue sections were blocked with 10% donkey serum at room temperature for 1 hour in PBS containing 0.03% Triton X-100. Fixed tissues were incubated overnight at 4 °C with respective primary antibodies to NOX1 (1:250), and NOX2 (1:250). Immunofluorescence staining was performed using donkey-antirabbit Alexafluor 594(1:1000) for NOX1 or NOX2. The specificity of each antibody staining was validated by excluding each primary antibody (negative controls) and visualized for any non-specific fluorescence.

2.6 Image Acquisition and Analysis

Slides with mounted coronal sections from the brain were imaged at 20x magnification using Leica Aperio Versa 200 digital pathology scanner. Control sections were used as reference for adjusting the exposure times and grey scale balance for optimal image quality, once set, these parameters were fixed and used for image acquisition of the

reminder of both control and experimental groups. Three channels were collected for each coronal section. Blue: 405 nm (DAPI), red: 594 nm (NOX1), and green: We then manually outlined the regions of interest in different brain structures and the fluorescence intensities in each brain region were quantitated using FLAreaQuantV1 algorithm (Leica Biosystems) and expressed as average fluorescence intensity/unit stained area. For each channel, we set a minimum intensity threshold value using control sections as reference that will exclude any background fluorescence caused by nonspecific binding of fluorescent secondary antibody, and the same threshold values were used to quantify both control and experimental groups. A maximum intensity threshold was also set to remove any oversaturation due to excess fluorescent dye. The algorithm outputs the area of positive staining for each brain region, the average intensity of channel, total area stained and total area analyzed. The final value [(total area stained/ total area analyzed) x the intensity] obtained in control groups was set as 100% and the values similarly obtained in experimental groups was expressed as percent change over control".

Figure 2.5: Leica Aperio Versa 200 fully automatic digital slide scanner with fluorescent microscope used in the present study to digitize the images of various brain regions and analyze the fluorescence intensities of NOX 1 and NOX2 proteins.

2.7 Superoxide Production

Superoxide ($O_{2.}^{-}$) levels in frontal cortex, hippocampus and thalamus were measured using dihydroethidium (DHE) following the method of Kim et al., [50]. Briefly, control and blast-induced animals immediately following blast were injected with 5mg/kg DHE (Molecular Probes, MA, dissolved in DMSO) i.p. and 24 hours after blast, animals were transcardially perfused first with PBS followed by 4% PFA, brains excised and 50 µm thin sections of different brain regions were prepared using Leica VT 1000S vibratome and mounted. DHE immunofluorescence in each region was visualized by digitizing the images using Leica Aperio Versa 200 slide scanner. Fluorescent intensities in each region were quantitated using AreaQuant software (Leica Biosystems) and average fluorescence intensity/unit area of control group was set as 100% and the changes in the intensities experimental groups was expressed as % over control.

2.8 Statistical Analysis

In this study we compared three Blast over pressure to find if there is any statistical significance between each group and we used T-test to find statistical increase in NOX level within the groups.

2.8.1 ANOVA

The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups, in this instance differences between 3 different BOPs (130 kPa, 180 kpa and 240 kpa). To determine which specific groups differed from each other, we used Tukey post hoc Test. This study helped us identify if there was any statistical significance between different Blast over pressure. Example We have three blast over pressure

130Kpa 180Kpa and 240Kpa this test would compare each group with each other i.e. 130 with 180, 130 with 240 and 240 with 180 and tell us which one of them is statistically significant from each other with given confidence interval of 95%.

2.8.2 T-Test

The independent t-test, also called the two sample t-test, independent-samples t-test or student's t-test, is an inferential statistical test that determines whether there is a statistically significant difference between the means in two unrelated groups. In relation to our study we use this test to find if there is statistical significance between any of Control and Blast at specific blast over pressure and time point i.e. If we have data for Blast at 130kpa at 24 hour we compare it to the control and using this test we prove that statistically the Blast and Control group are different and that increase we found is statistically significant**.**

CHAPTER 3

RESULTS

As noted in Chapter 1, the rationale of this study was based on our recent publication [13] which reported cell-specific responses of NOX isoform expression 4h post injury in animals exposed to mild/moderate blast over pressure (180 kPa). We extended this study to identify NOX expression as function of blast over pressure and function of post injury time and also identify if NOX varies in different cerebral region. Lastly we also measured the downstream effect of increase in NOX.

3.1 Protein Levels of NOX1 and NOX2 Isoforms Increase As a Function of Overpressures in Blast TBI

Previous studies in this laboratory identified increased oxidative and nitrosative stress factors in the cerebral cortex in rats exposed to mild-bTBI [48, 49] and that NOX1 and NOX2 isoforms display a cell-specific increase at moderate blast pressure (180 kPa) 4hours post injury [13]. This study further evaluated the effect of different blast overpressures on the protein levels of NOX1 and NOX2 isoforms at 3 different blast overpressures namely 130 kPa, 180 kPa and 240 kPa 24hours following blast injury.

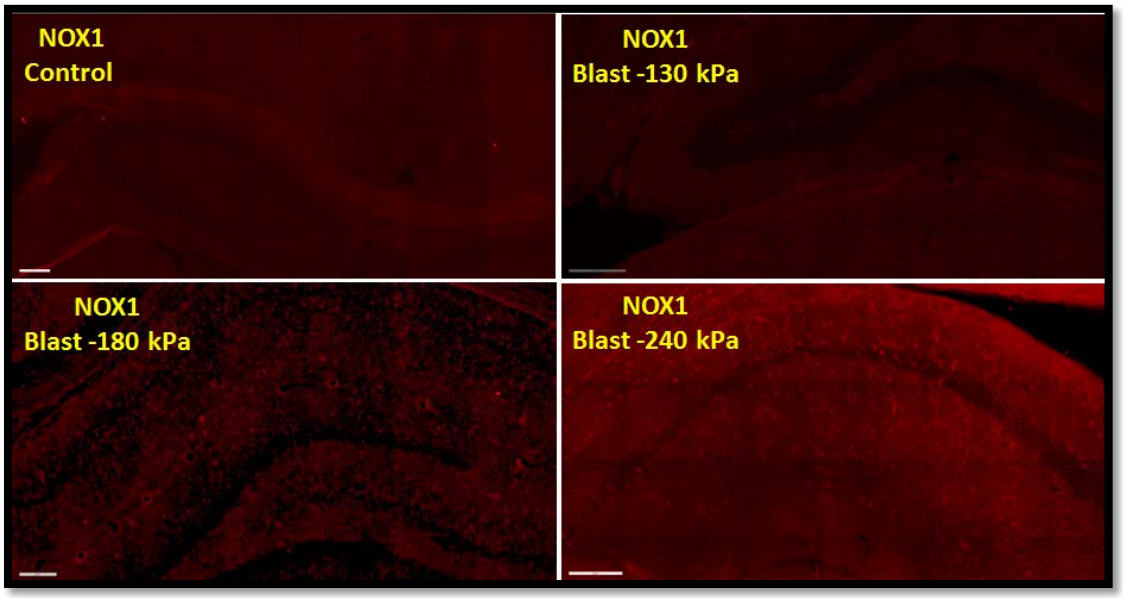

Figure 3.1: Protein level of NOX1 isoform (red) showing a biphasic response in hippocampus as a function of overpressures in animals exposed to blast TBI. Note there was no significant change at 130 kPa, whereas at 180 kPa and 240 kPa there was a progressive increase in NOX1 protein. Note: The scale bar of each image at this magnification was 300 µm. The size of the image and the anatomical location of the region slightly vary in each image selected. However, these are images were presented for the illustration purpose. During the process of quantification of fluorescent image intensities, we used images with identical magnification for marking annotations to identify each region.

Immunofluorescence staining and subsequent analysis (for analysis refer chapter 2.6) of the fluorescence intensities of NOX1 and NOX2 in different brain regions showed a biphasic response. In animal groups exposed to 130 kPa did not show any significant change in NOX1 protein levels whereas in animal groups exposed to 180 kPa showed a significant increase (Frontal cortex, hippocampus and thalamus 84%, 81% and 68% ± 5% respectively, $p<0.05$) in frontal cortex, hippocampus and thalamus. Interestingly, in animal groups exposed to higher blast over pressure of 240 kPa showed a further increase in frontal cortex (Frontal cortex, hippocampus and Thalamus showed 159%, 115% and 110%, ± 15% respectively, $p<0.05$), (Figures 3.1 and 3.2). The pattern of changes in the protein levels of NOX2 also displayed a changes as that of NOX1 (Figure 3.3)

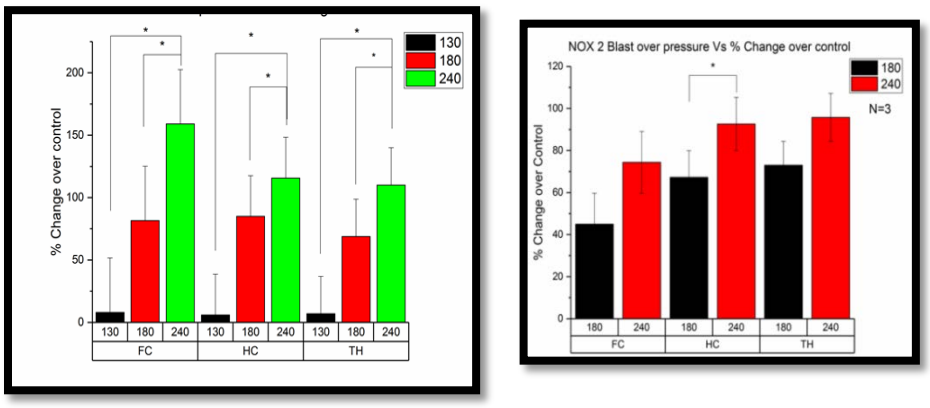

Figure 3.2: Quantification of protein levels of NOX1 (left panel) and NOX2 (right panel) display a similar pattern of change as a function of overpressures in animals exposed to blast injury. Frontal Cortex (FC) , Hippocampus (HC) and Thalamus (TH) showed 159, 115 and 110 % change over control at 240Kpa, at 180 Kpa they showed 84, 81 and 68 % change over control and at 130Kpa it showed 8, 6 and 7 % change over control for NOX-1 study. For NOX2 study Frontal Cortex (FC), Hippocampus (HC) and Thalamus (TH) showed 159, 115 and 110 % change over control at 240Kpa, at 180 Kpa they showed 74, 92 and 95 % change over control and at 180Kpa it showed 45, 67 and 73 % change over control

3.2 Protein Levels of NOX1 and NOX2 Isoforms Display a Biphasic Response as a Function of Time Post Exposure in Blast TBI

We next examined whether there are any time-dependent changes in the protein levels of NOX isoforms in different brain regions. For this we elected animal groups exposed to 240 kPa BOP and compared the changes with control groups at 3 time points namely 4h, 24h and 7 days post-injury. As noted in Figure 3.3, both NOX1 and NOX2 protein levels displayed a similar pattern of changes in which there was a progressive increase in their levels at 4 and 24 h post injury, whereas the levels of both NOX1 and NOX2 returned back to control levels by 7 days.

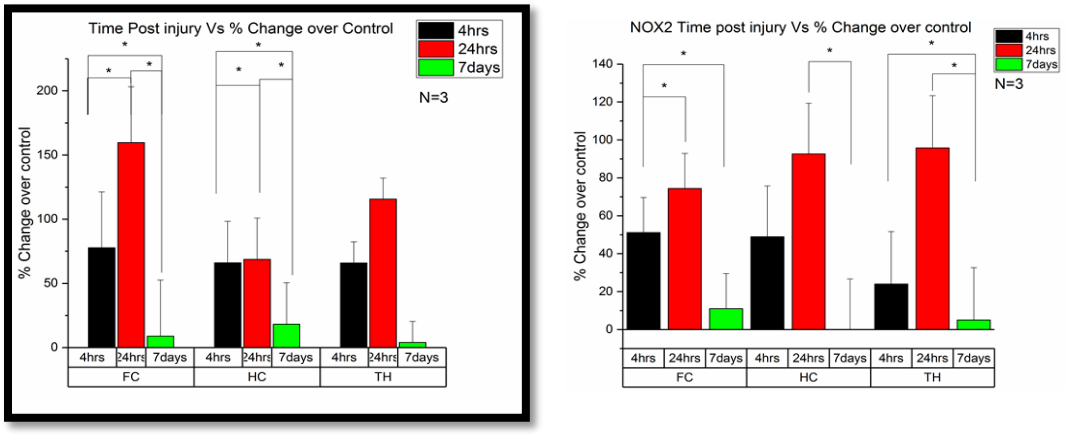

Figure 3.3: Quantification of protein levels of NOX1 (left panel) and NOX2 (right panel) display a biphasic response of change as a function of time in animals exposed to blast injury. % change over control at 4hr 24hr and 7 days for Frontal cortex (FC)are 77% ,159% ,9% and for Hippocampus (HC) is 66,115 and 18% and for Thalamus (TH) its 65, 68 and 4% for NOX-1. NOX-2 showed % change over control at 4hr 24hr and 7 days for FC are 51% ,74% ,11% and for HC is 48,92 and 0% and for TH its 24, 95 and 5%

3.3 NOX1 Expression Display Differential Changes in Different Brain Regions

As noted in previous sections, in a recent study, we displayed a differential response of NOX1 expression changes in animals exposed to moderate blast (180 kpa) in the acute phase of the injury (4h) [13]. In the present study, we therefore examined whether higher BOP (240 kPa) at longer time (24h) post injury also show a regional variation in NOX1 expression. Results show that NOX1 levels were significantly higher in frontal cortex compared to hippocampus (Figure 3.4). Interestingly, thalamus displayed a least degree of change in NOX1 expression.

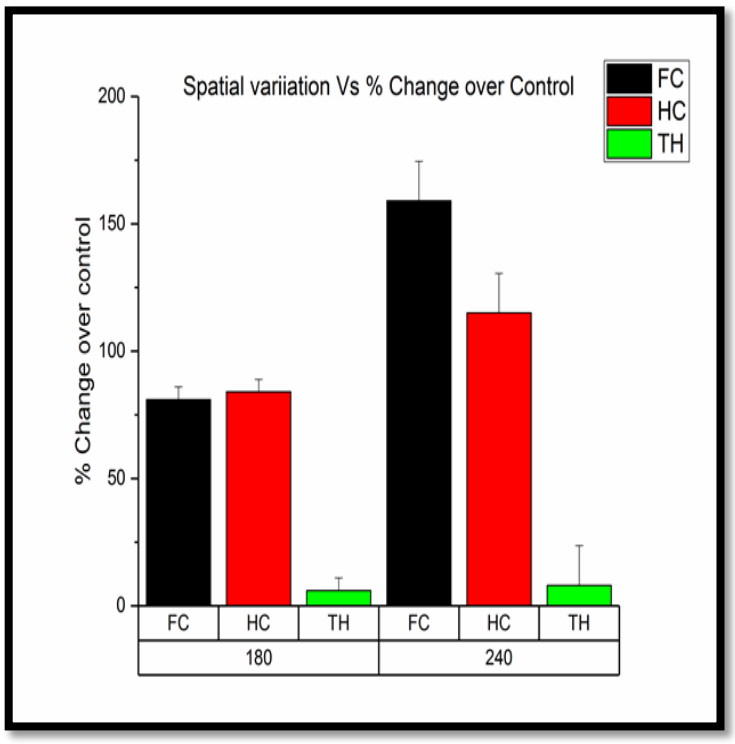

Figure 3.4: Quantification of protein levels of NOX1 display a regional variation in animals exposed to 240 kPa blast injury. Frontal cortex, Hippocampus and Thalamus show 84%, 81 % and 6% change over control at 180 Kpa and at 240 Kpa they show 159%, 115% and 8% change over control.

3.4 Primary Blast Increases Superoxide Levels in Different Brain Regions

Several studies reported that one of the downstream effects of NOX activation is the production of superoxide anion (O_2^-) [44, 51]. We therefore examined the *in vivo* levels of superoxide 24h post-injury in frontal cortex, hippocampus and thalamus in animals exposed to 180 kPa using DHE. Results showed that superoxide levels were significantly increased in frontal cortex, hippocampus and thalamus in animals exposed to blast injury ($p<0.05$)(Figure3.5).

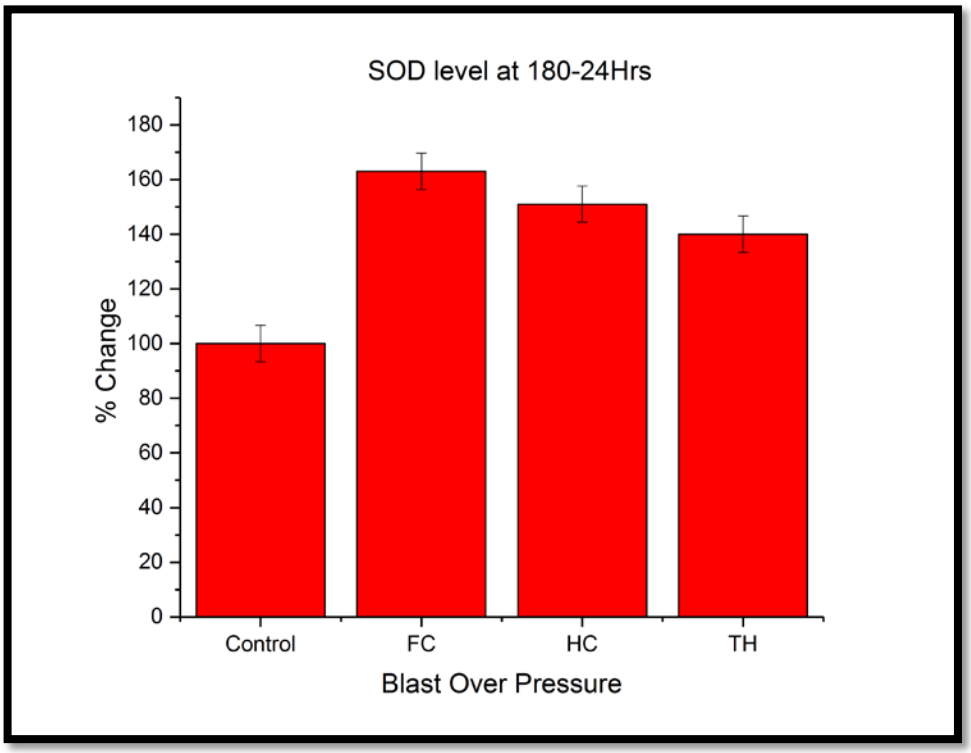

Figure 3.5: Quantification of superoxide levels in frontal cortex, hippocampus and thalamus 24 hours post-injury in animals exposed to 180 kPa. $P<0.05$

3.5 Primary Blast Shows a High Tendency to Cause Oxidative Damage and Lipid Peroxidation Products in Brain Proteins

Several studies reported that different forms of TBI at different times post injury cause structural changes in several brain proteins by incorporating adducts of lipid peroxidation [39, 52-54]. One such peroxidation adduct of lipids include the formation of 4-hydroxynonenal (4-HNE) [55-58]. 4-HNE formation process when oxidants such as free radicals attack various lipids containing carbon double bonds of unsaturated fatty acids and form aldehyde products such as 4-HNE and these products chemically react with proteins to form adducts [59]. Studies have also shown that superoxide is one of the free radicals that is able to oxidize lipids to aldehyde products such as 4-HNE[51, 60, 61]. We therefore examined whether moderate blast injury (180 kPa) results in increased superoxide levels 24h post injury time. As presented in Figure 3.6, immunoblot analysis was performed for ~~of~~ 4HNE adducts in proteins and the quantity of density in each band (noted in Section 2.4) was normalized to actin (housekeeping gene) protein bands. We identified HNE products in a major band corresponding to molecular weights of 70 kDa which showed a strong tendency towards an increase in cerebral hemisphere, however such changes were not significant (Figure 3.6).

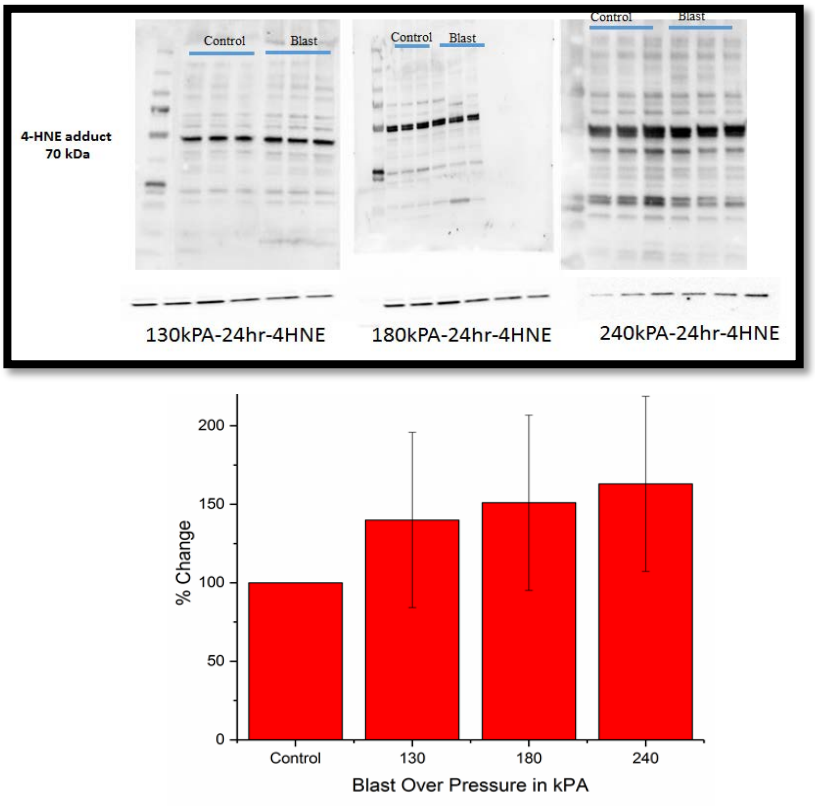

Figure 3.5: Primary blast displays a strong tendency to increase 4-HNE protein adducts. Immunoblot analysis of 4-HNE in lysates from cerebral hemispheres 24h after blast at 180 kPa blast over pressure is shown. A prominent protein of 70 kDa show a strong tendency to increase.

CHAPTER 4

DISCUSSION

This study demonstrates that protein levels of superoxide producing enzymes NOX1 and NOX2 were significantly increased varying overpressures in blast TBI. Such increased NOX protein levels correlated with increased levels of superoxide and strong tendency towards increased HNE adduct formation in 70 kDa protein. Taken together, my study indicate that NOX-mediated oxidative stress shows a BOP-dependent changes in blast TBI.

Several reports from our laboratory indicated that blast injury displays a unique pathology compared to other forms of TBI (such as blunt TBI) wherein the injury appears uniform throughout the brain structures because of the uniform propagation of the shockwave through skull and the brain [13, 23, 25, 48, 54, 62-66].

Oxidative stress has been implicated as a major pathological factor in many forms of TBI which operates at several levels including the increased activation of free radical producing enzymes or downregulation of antioxidant defense mechanisms [39, 53, 54, 67]. NADPH oxidase (NOX) is a superoxide producing enzyme and different isoforms of NOX, including NOX1, NOX2 and NOX4 have been identified in brain [44]. Studies reported increased activation of different isoforms of NOX in various forms of TBI. Accordingly, controlled cortical impact (CCI) in both rat and mice models showed a robust increase in NOX isoforms [68, 69]. Likewise, increased NOX expression was also found in different animal models of fluid percussion injury [27, 46, 70]. Together these studies highlight the critical role of NADPH oxidase in the pathology of TBI.

Studies on the role of NOX in the pathophysiology of blast TBI are limited. Studies by [32, 71, 72] reported increased NOX expression in animals exposed to blast TBI. Studies from our laboratory also reported increased NOX protein as a pathological factor in the oxidative stress-mediated injury pattern in bTBI [48, 49, 54]. Additionally, recent report by Rama Rao et al, [13] showed cell-specific changes in NOX isoform expression in vulnerable brain regions in 4h after moderate (180 kPa) blast injury. Accordingly, all the regions of the brain from rostral to caudal areas including frontal cortex, striatum, somatosensory cortex, hippocampus, thalamus as well as cerebellum displayed increased NOX expression. Additionally, neurons appeared to show highest increase in NOX compared to astrocytes

and microglia [13]. These studies together established that NOX-mediated oxidative stress is one of the major pathological factors in bTBI.

While the above studies strongly establish the role of NOX activation in the pathophysiology of bTBI, studies thus far have not examined the NOX changes as a function of different blast over pressures and as a function time post injury. These are the two important parameters that have high pathophysiological significance in blast TBI, since, 1). The increase in the NOX expression with the increasing overpressures would predict different degree of oxidative damage that can possibly occur in bTBI of varying severity, i.e., mild to-moderate-severe intensities; 2). Identifying the time course of NOX-mediated oxidative stress would predict any possible recovery of the injury pathology during the course of bTBI.

Therefore, in the present study, a progressive increase in NOX protein levels with increasing blast overpressures strongly suggests that higher the intensity of BOP, higher will be the oxidative damage occurring in bTBI. This is reasonable that the degree of structural damage encountered by brain at higher intensities of shockwave will be expected to be higher. In fact, a recent study in our laboratory carried out by Kuriakose et al., [25]indeed displayed a progressive increase in blood brain barrier (BBB) permeability with increasing overpressures in various brain regions, as shown by increased extravasation of Evans blue and sodium fluorescein. Our current study is in correlation with studies by Kuriakose et al., and together indicates that vascular permeability changes and oxidative stress follow a pattern of change consistent with increasing blast overpressures. However, noteworthy that NOX prorein levels did not change by single blast at 130 kPa suggesting that this degree of overpressure is insufficient to exert oxidative damage in bTBI. This

however does not preclude that no other pathological changes manifest at this BOP, since Kuriakose et al., did find a significant increase BBB permeability in frontal cortex at this BOP which again suggest that different varieties of injury factors activate differentially at different intervals and/or at different blast overpressures.

The time course of changes in NOX1 protein levels display a biphasic response in which 4hours and 24 hours show a progressive increase in its expression whereas 7 days post injury the protein levels were completely restored normal as that of controls. It is interesting to note again that we found similarities between temporal profile of changes in BBB permeability and NOX protein expression (in the current study) wherein studies by Kuriakose et al., [25] found a complete restoration of BBB integrity within 24 hours following blast injury in animals exposed to 180 kPa BOP. The reason for complete restoration of the NOX protein levels 7 days post-injury is not known. However, it is highly likely that some compensatory mechanisms may be operative to neutralize the NOX protein expression and subsequent oxidative mechanisms. One such compensatory mechanisms may be activation of antioxidant enzymes which are involved in scavenging superoxide free radicals that are known to damage macromolecules including membrane lipids, proteins, RNA and DNA or enhancing the glutathione levels by activating enzymes of glutathione metabolism. Indeed, studies have shown such increase in glutathione synthesizing enzymes glutathione peroxidase, as well as antioxidant scavenging enzyme catalase 7 days post-injury in a rat model of controlled cortical contusion model [73]. However, whether or not antioxidant capacity increased in blast TBI models needs to be determined.

In the present study, levels of increase in superoxide well correlated with the increased protein levels of NOX, strongly suggesting that NOX increase following blast injury produces excessive levels of superoxide. Superoxide is a major free radical produced in the brain by a variety of reactions, including disturbances in mitochondrial oxidative phosphorylation, increased production of arachidonic acid as a consequence of activation of phosphilipase A2 (PLA2), activation of xanthine oxidase as well as by the activation of NOX [74-77]. Increased superoxide production has been shown in different models of TBI [77-81].

While we find increased NOX protein levels and subsequent increased superoxide free radicals in the present study, the levels of 4HNE adducts showed only a strong trend towards the increase in protein of molecular weight approximately 70 kDa but did not show statistically significant changes. Precisely why there was no statistical significance between control and animals exposed to blast injury is not known. It is possible that the methodological sensitivity of the assays, in this instance determination of 4-HNE by Western blots may not be sensitive enough as to show a significant change. Alternatively, it is possible that lipid peroxidation reaction and subsequent 4-HNE formation may not have achieved a threshold as to show significant changes at 24 hours following blast.

In summary, our studies demonstrate that NOX isoforms show a progressive increase in their protein expression as a function of increasing overpressures in blast TBI. Additionally, there was a biphasic response observed in the temporal profiles of increase in NOX protein levels. Further, increased superoxide production observed in the present study correlated well with the increase NOX protein levels as a function of time. Together these

studies indicate that NOX protein changes and associated downstream effects of NOX increase follow a pattern of progressive increase as a function of varying overpressures and time course of blast injury. Targeting NOX-mediated oxidative damage may have a therapeutic benefit to ameliorate pathology associated with blast TBI.

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